# Microfilariae of the filarial nematode Litomosoides sigmodontis exacerbate the course of lipopolysaccharide-induced sepsis in mice

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# Microfilariae of the Filarial Nematode *Litomosoides sigmodontis* Exacerbate the Course of Lipopolysaccharide-Induced Sepsis in Mice▽

Marc P. Hübner,[1]* Bastian Pasche,[2] Svetoslav Kalaydjiev,[3]† Peter T. Soboslay,[1] Andreas Lengeling,[2] Hartwig Schulz-Key,[1] Edward Mitre,[4] and Wolfgang H. Hoffmann[1]

*Institute of Tropical Medicine, University of Tübingen, 72074 Tübingen, Germany[1]; Helmholtz Centre for Infection Research, 38124 Braunschweig, Germany[2]; Institute for Medical Microbiology, Immunology, and Hygiene, Technical University of Munich, 81675 Munich, Germany[3]; and Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814[4]*

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**Helminths facilitate their own survival by actively modulating the immune systems of their hosts. We investigated the impacts that different life cycle stages of the rodent filaria *Litomosoides sigmodontis* have on the inflammatory responses of mice injected with sublethal doses of lipopolysaccharide (LPS). Mice infected with female adult worms from prepatent infections, worms which have not yet started to release microfilariae, developed lower levels of proinflammatory cytokines in the peripheral blood after LPS challenge than sham-treated controls, demonstrating that female adult worms can mitigate the innate immune response. The presence of microfilariae in mice, however, through either direct injection or implantation of microfilaria-releasing adult female worms, turned the LPS challenge fatal. This lethal outcome was characterized by increased plasma levels of gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin 12 (IL-12), and IL-6, greater numbers of macrophages and granulocytes in the peripheral blood, and decreased body temperatures in microfilaria-infected mice. Microfilaria-infected mice deficient in IFN-γ receptor and TNF receptor 1 had increased survival rates after LPS challenge compared to immune-competent mice, suggesting that microfilariae worsen LPS-induced sepsis through actions of IFN-γ and TNF-α. In summary, we have demonstrated that infection of mice with *L. sigmodontis* female adult worms from prepatent infections protects mice injected with LPS whereas microfilariae worsen LPS-induced sepsis through the induction of proinflammatory cytokines and upregulation of granulocytes, NK cells, and monocytes in the peripheral blood.**

Infections with helminths often cause chronic disease with a wide spectrum of clinical manifestations, ranging from asymptomatic to severe pathology. By actively suppressing the inflammatory responses of their hosts, helminths facilitate their own survival (26). This suppression will not only permit parasite persistence at high infection levels but also avoid pathological changes to the host. Amazingly, the presence of only one female adult worm of the rodent filarial nematode *Litomosoides sigmodontis*, an animal model for human filariasis (1, 18), is sufficient to prolong the viability of their progeny, the microfilariae, in the host (19). Although microfilariae given alone provoke a Th1 response in their hosts (25), adult filarial worms typically induce a Th2 immune response characterized by high levels of interleukin 4 (IL-4), IL-5, IL-10, IL-13, and immunoglobulin E as well as an augmentation of mast cells, eosinophils, and basophils (26).

In addition, helminths induce a stage of hyporesponsiveness in their hosts (14), likely due to a targeted immune suppression by IL-10, transforming growth factor β, regulatory T cells, and alternatively activated macrophages (12, 16, 23, 33, 36, 37). Recently, this immunomodulatory capacity of parasitic worms or their excretory/secretory products has been effectively utilized for therapeutic purposes in patients with inflammatory bowel disease (35) as well as in mouse models of autoimmune or allergic diseases (22, 24, 27, 34, 40, 41). To date, however, no studies have evaluated the effect helminths may have on the innate immune response to sepsis.

In this study, we examined whether *L. sigmodontis* at different life cycle stages has the ability to alter the course of disease in lipopolysaccharide (LPS)-induced sepsis in mice, a widely used model for septic shock syndrome. To investigate the effect that *L. sigmodontis* has on LPS-induced sepsis in mice, we examined cytokine concentrations in mice infected with microfilariae, male adult worms, and female adult worms from prepatent (microfilaria negative), patent (microfilaria positive), or postpatent (microfilaria negative) infections for 48 h after LPS challenge. Microfilaria-injected mice were also evaluated for changes in peripheral cell populations and body temperature. To specifically test the roles of various cell surface receptors known to be involved in LPS binding, macrophage receptor with collagenous structure (MARCO) knockout mice (9) as well as tumor necrosis factor receptor 1 (TNFR1) (32) and gamma interferon receptor (IFN-γR) knockout mice were given LPS challenge in the presence or absence of microfilariae.

## MATERIALS AND METHODS

**Mice and parasites.** C57BL/6 mice were originally obtained from Harlan-Winkelmann (Borchen, Germany). Breeding pairs of IFN-γR knockout mice (B6.129S7-Ifngr1tm1Agt$^{-/-}$) were obtained from Jackson Laboratories, Bar

* Corresponding author. Present address: Dept. of Microbiology and Immunology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814. Phone: (301) 295-3447. Fax: (301) 295-3773. E-mail: mhubner@usuhs.mil.

† Present address: Faculty of Life Sciences, The University of Manchester, Manchester M13 9PT, United Kingdom.

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Harbor, ME, whereas TNFR1 (TNFRp55$^{-/-}$) and MARCO knockout mice were kindly provided by K. Pfeffer, University of Düsseldorf, Germany, and K. Tryggvason, Karolinska Institute, Sweden, respectively. All knockout strains have been backcrossed onto a C57BL/6 background for at least 10 generations. The animals were maintained and further bred in our facility in individually ventilated cages. The C57BL/6 strain was chosen because this is the strain commonly employed in LPS sepsis models (39) and because all the knockout mice had this genetic background. Although C57BL/6 mice are not fully permissive for *L. sigmodontis* infection, they do sustain the infection for several weeks (19).

All experiments were conducted with mice of both genders, evenly distributed over the different groups, and the mice were 4 to 6 weeks of age at the time of infection.

Adult worms of *Litomosoides sigmodontis* were isolated from the pleural cavities of previously infected jirds (*Meriones unguiculatus*) or cotton rats (*Sigmodon hispidus*) under sterile conditions. The worms were isolated at different time points from heavily infected jirds and cotton rats and categorized, according to the presence of microfilariae in the peripheral blood samples of the donor animals, as worms from prepatent (microfilaria negative), patent (microfilaria positive), or postpatent (microfilaria negative) infections. Microfilariae were isolated from the peripheral blood samples of infected hosts and purified using a Percoll gradient as described previously (6).

**Infection of mice.** Mice were anesthetized with a mixture of 15 mg/kg of body weight of xylazinehydrochloride (Rompun; Bayer Vital, Leverkusen, Germany) and 100 mg/kg of body weight of esketaminehydrochloride (Ketanest; Parke-Davis, Berlin, Germany). Fifty thousand microfilariae suspended in 20 to 50 μl RPMI 1640 containing 25 mM HEPES and L-glutamine (Gibco, Invitrogen Corporation, Auckland, New Zealand) were injected into the jugular vein. Implantation of five living adult worms in RPMI occurred intraperitoneally (i.p.). Control groups were sham treated, i.e., they received the same volumes of RPMI by use of identical surgical procedures.

Peripheral microfilaremia was determined 1 hour after injection of microfilariae and 10 days after implantation of female adult worms by first treating 30 μl of a blood sample with 1 ml fluorescence-activated cell sorting (FACS) lysing solution (BD Biosciences, San Jose, CA) and then counting all microfilariae by microscopy.

One day after microfilaria infection or 10 days after implantation of adult worms, mice were i.p. injected with 4 mg/kg of body weight of *Escherichia coli* LPS (026:B6; Sigma, St. Louis, MO). Controls received the corresponding amounts of phosphate-buffered saline (PBS).

The survival of mice was observed for 48 h after LPS challenge, as no mice died after this time point. Peritoneal fluid smears of infected mice which had no detectable microfilariae in the peripheral blood during the course of the experiment were examined at the end of the experiment to check the fecundity of implanted female worms.

**Determination of cytokines, chemokines, and cell populations.** Peripheral blood (300 μl per sample) was collected by retro-orbital bleeding using heparinized microtubes (BD Microtainer, Franklin Lakes, NJ), and plasma was prepared according to the manufacturer's recommendations. Blood was collected 1 hour after microfilaria infection or 10 days after implantation of adult worms as well as 1 and 6 hours after LPS challenge. Plasma cytokine concentrations were measured using a commercially available enzyme-linked immunosorbent assay for quantification of IFN-γ (AN-18), TNF-α (mono/mono), IL-6, IL-10, and IL-12 (p40) (OptEIA mouse set; BD Biosciences, San Diego, CA).

Plasma concentrations of monocyte chemoattractant protein 1 (MCP-1), monokine induced by IFN-γ (MIG), granulocyte monocyte colony stimulating factor (GMCSF), macrophage inflammatory protein 1α (MIP-1α), IL-1α, and IL-1β were analyzed 6 hours after LPS challenge by using a mouse cytokine Twenty-Plex kit (BioSource International Inc., Camarillo, CA), based on Luminex technology. All samples were threefold diluted, and the assay was carried out according to the manufacturer's instructions. Measurement was performed using the LiquiChip workstation (Qiagen, Hilden, Germany), and data were analyzed with LiquiChip Analyzer 1.0 software (Qiagen, Hilden, Germany).

Cell populations were examined 6 hours after LPS injection in heparinized peripheral blood by FACS analysis (20) in the German Mouse Clinic. This experiment was conducted separately to avoid nonspecific effects due to repeated bleeding. After lyses of erythrocytes with 0.17 M $NH_4Cl$-Tris buffer (pH 7.45), cells were washed with FACS staining buffer (PBS, 0.5% bovine serum albumin, 0.02% sodium azide, pH 7.45). Leukocytes were incubated for 20 min with anti-Fc receptor antibodies (clone 2.4G2; PharMingen, San Diego, CA). Afterwards, cells were stained with fluorescence-labeled antibodies (CD4 clone RM4-5, CD8α clone 53-6.7, CD8β clone H35-17.2, Gr-1 RB6-8C5, CD11b M1/70, and CD49b DX5; PharMingen). Dead cells were excluded by propidium iodide staining. Twenty-five thousand cells were acquired with a CyAn ADP analyzer (DakoCytomation, Ft. Collins, CO).

**Measurement of temperature.** The rectal temperatures of mice were measured before and 6 hours after LPS challenge.

**Statistical analysis.** To analyze differences in concentrations of cytokines and chemokines between the differently treated groups, data were logarithmically transformed to stabilize the variance of data [log (pg/ml + 0.5)]. Differences between groups were tested for significance using the Kruskal-Wallis test, followed by Dunn's post hoc multiple comparisons. Statistical differences in concentrations of cytokines after LPS injection in microfilaria-infected and control mice were analyzed using the Mann-Whitney U test. Data from FACS analysis were stabilized by converting percentage values to arcsin values [arcsin (square root of values in percentage/100)], and significance was determined by the Wilcoxon/Kruskal-Wallis test. $P$ values of $<0.05$ were considered significant. Cytokine and chemokine levels as well as body temperature are presented as medians. Survival data are shown as Kaplan-Meier graphs and were analyzed by the log rank test.

## RESULTS

**Presence of microfilariae in the peripheral blood of wild-type mice causes sublethal LPS injections to be fatal.** All mice infected with female adult worms from prepatent and postpatent infections or with male adult worms survived the LPS challenge (Fig. 1A). Blood and peritoneal smears from these mice demonstrated no circulating microfilariae and few, if any, microfilariae in the peritoneum, confirming the postpatent and prepatent stages of female adult worms. In contrast, all mice infected with microfilariae or female adult worms from patent infections, immediately releasing large numbers of microfilariae into the peripheral blood (about 50 to 200 per 100 μl), died within 2 days after injection of LPS (Fig. 1A), with most deaths occurring in the first 24 h after LPS challenge (Fig. 1 B). The survival rates among the sham-treated controls were between 69 and 92% after LPS challenge (Fig. 1 A) and were consistent in the different experiments. All animals of the PBS control group survived the experiment, irrespective of whether they were infected with *L. sigmodontis* at different developmental stages or not (Fig. 1 A).

In contrast to the wild-type mice, all IFN-γR knockout mice and 80% of the TNFR1 knockout mice, whether infected with *L. sigmodontis* microfilariae or not, survived the LPS challenge (Fig. 1C). Three out of five microfilaria-infected mice that were deficient in MARCO, a type A scavenger receptor that binds LPSs and which is expressed on macrophages, dendritic cells, and microglia cells (15), died after LPS challenge, whereas all noninfected MARCO knockout mice survived the LPS challenge (Fig. 1C).

**Microfilaremia increases cytokine concentrations during sepsis, whereas implanted female adult worms from prepatent infections reduce them.** The injection of a sublethal amount of LPS in mice resulted in significant increases of all measured cytokine concentrations compared to those for the PBS controls (data not shown). Preliminary time course experiments revealed that TNF-α levels peaked 1 hour after LPS injection, whereas the remaining cytokines were increased 6 hours after LPS challenge (data not shown). As such, plasma cytokine levels were measured at 6 hours for all cytokines except TNF-α, which was measured 1 h after LPS injection. Non-parasite-exposed PBS control groups showed no change in plasma cytokine concentrations over the course of the experiment (data not shown).

While implantation of *L. sigmodontis* male worms and female adult worms from postpatent infections resulted in no

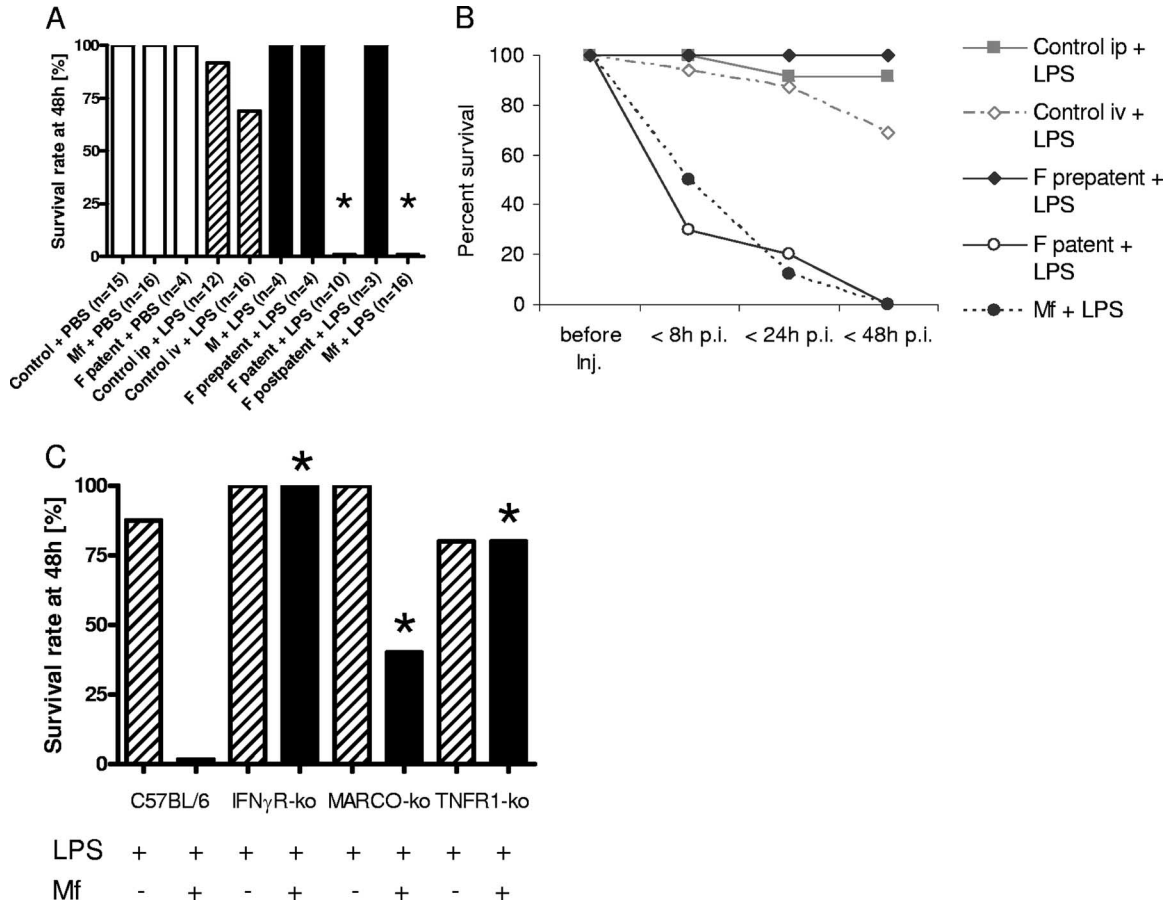


FIG. 1. Percent survival of C57BL/6 mice 48 h after injection with PBS or LPS (A) and kinetics of survival before and 8, 24, and 48 h after LPS injection (B). Mice were infected with microfilariae (Mf), male (M) or female (F) worms from prepatent or patent infections. Controls got the surgical procedure used for the microfilaria (intravenous) or worm (i.p.) infection. (C) Percent survival of microfilaria (Mf+)-infected wild-type C57BL/6 ($n = 8$ per group), IFN-γR ($n = 3$ per group), MARCO ($n = 5$ per group), and TNFR1 ($n = 5$ per group) knockout mice and controls (Mf−) 48 h after LPS challenge. All experiments were repeated at least once, except for the survival data with implanted worms from prepatent or postpatent infections and the experiments with IFN-γR knockout mice. Survival data were analyzed as Kaplan-Meier graphs by the log rank test and showed significant differences between infected mice and the LPS-treated control groups (*, $P < 0.05$). Panel C shows significant differences in survival rates after LPS and microfilaria infection between C57BL/6 mice and IFN-γR, MARCO, or TNFR1 knockout mice (*, $P < 0.05$).

significant differences in plasma cytokine concentrations in response to LPS, implantation of female adult worms from prepatent infections resulted in decreased concentrations of the proinflammatory cytokines IFN-γ, TNF-α, and IL-12p40 and in equivalent amounts of IL-10 in the peripheral blood samples of mice compared to the levels for sham-treated controls after LPS challenge (Fig. 2).

In contrast, the presence of adult worms which released microfilariae in mice resulted in significantly increased IFN-γ and IL-12p40 concentrations after LPS challenge compared to those for sham-treated controls, whereas concentrations of TNF-α and IL-6 were not significantly elevated (Fig. 2). Mice infected with worms from patent infections had significantly higher IL-10 concentrations after LPS challenge than sham-treated mice (Fig. 2).

Microfilariae were confirmed as the cause of this pronounced inflammatory response to LPS, as the direct injection of microfilariae into mice (in the absence of any adult worms) resulted in enormous increases in proinflammatory cytokines after LPS challenge compared to the levels for sham-treated animals (Fig. 3). Most notably, the concentrations of IFN-γ increased 136-fold, whereas TNF-α and IL-6 concentrations showed 40-fold increases (Fig. 3). The cytokines IL-12p40 and IL-10 were significantly elevated as well (Fig. 3). IL-1α and -β were analyzed together with the chemokines by the Luminex method 6 hours after LPS/PBS injection, and the levels were significantly higher after LPS treatment in microfilaria-infected mice than in the sham-treated controls (Table 1). In addition, microfilaria-infected mice showed significant increases in the chemokine concentrations of MCP-1 (CCL2), MIG (CXCL9), MIP-1α (CCL3), and GMCSF 6 hours after LPS injection compared to sham-treated controls (Table 1).

Interestingly, in the absence of LPS challenge, the injection of microfilariae induced low concentrations of IFN-γ, IL-12p40, and the chemokine MIG in the peripheral blood samples of mice which were treated with PBS (Table 1), but there were no differences from noninfected mice in IL-6, IL-10, or TNF-α concentrations (data not shown).

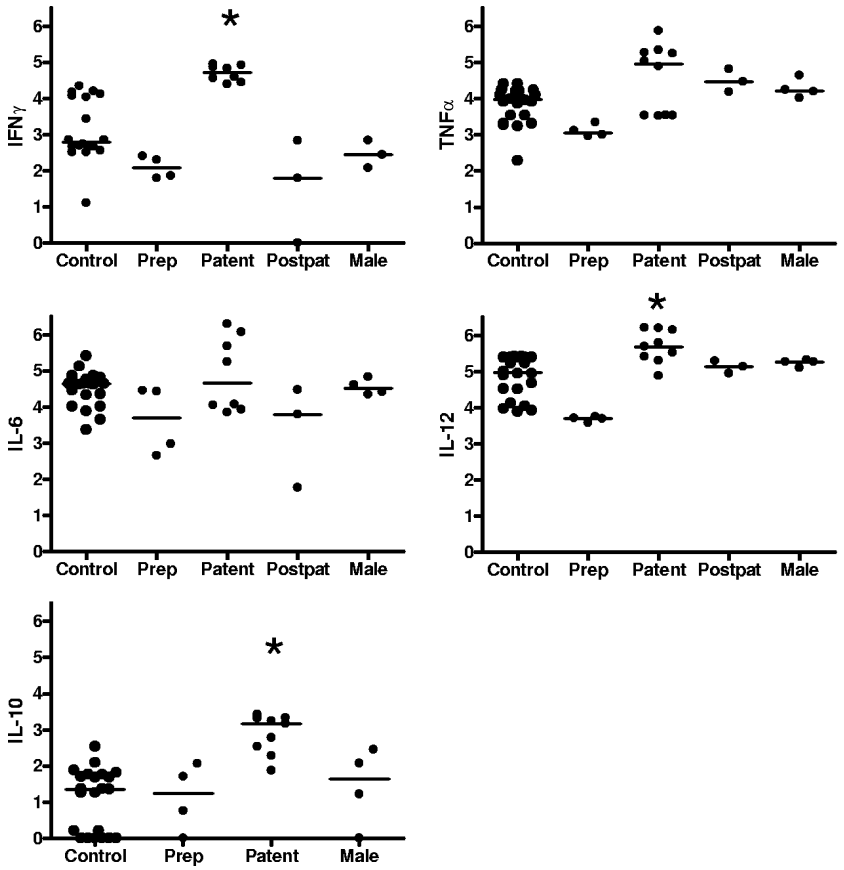


FIG. 2. Cytokine production [log (pg/ml + 0.5)] of infected or sham-treated C57BL/6 mice 6 hours (IFN-γ, IL-6, IL-12, and IL-10) and 1 hour (TNF-α) after LPS challenge. Mice were infected with female adult worms from prepatent ($n = 4$), patent ($n = 8$ to 10), and postpatent ($n = 3$) infections or male worms ($n = 4$), while the controls ($n = 22$) got the same surgical treatment but no worms. The data with worms from patent infections and control mice were obtained from two independent experiments. Significant differences between infected mice and the control group were analyzed by the Kruskal-Wallis test, followed by Dunn's post hoc multiple comparisons (*, $P < 0.05$).

**Microfilariae worsen LPS-induced sepsis through actions of IFN-γ and TNF-α.** To specifically test the roles of various cell surface receptors known to be involved in LPS binding, MARCO knockout mice (9) as well as TNFR1 (32) and IFN-γR knockout mice were given LPS challenge in the presence of microfilariae. While the MARCO-deficient, TNFR1-deficient, and IFN-γR-deficient mice infected with microfilariae developed increases in circulating levels of the proinflammatory cytokines IFN-γ, TNF-α, IL-6, and IL-12p40 after LPS challenge compared to sham-treated controls, some of these levels in microfilaria-infected TNFR1-deficient and IFN-γR-deficient mice were substantially lower than those that occur after LPS challenge of immunocompetent microfilaria-infected mice (Fig. 4).

Six hours after LPS challenge of microfilaria-infected mice, the concentrations of circulating IFN-γ were significantly lower in TNFR1-deficient mice, but not IFN-γR- or MARCO-deficient mice, than in immunocompetent mice (Fig. 4). Likewise, the concentrations of IL-6 in all knockout mice infected with microfilariae were decreased compared to those in the immune-competent mice 6 hours after LPS injection, with the reductions in TNFR1-deficient mice reaching statistical significance (Fig. 4).

Additionally, 1 hour after LPS challenge, IFN-γR-deficient mice infected with microfilariae had reduced levels of TNF-α compared to immune-competent mice, whereas TNFR1-deficient mice had slightly increased TNF-α concentrations (Fig. 4). IL-12p40 concentrations in TNFR1- and IFN-γR-deficient mice showed a tendency to be reduced compared to those in the immune-competent mice (Fig. 4).

In contrast to the results for proinflammatory cytokines, there was no difference in IL-10 concentrations between immune-competent mice and any of the knockout mice, regardless of their infection statuses after LPS challenge (Fig. 4).

**Increased numbers of granulocytes and monocytes in microfilaria-injected mice.** LPS challenge of microfilaria-infected mice resulted in a higher frequency of $CD11b^+$ $Gr\text{-}1^-$ monocytes than LPS challenge of uninfected mice as determined by flow cytometry (Fig. 5). In comparison to those in infected or noninfected PBS-treated mice, the frequencies of $CD4^+$, $CD8\alpha^+$, and $CD8\beta^+$ T cells were significantly reduced in LPS-challenged microfilaria-infected mice (Fig. 5).

The LPS challenge on its own resulted in decreased frequencies of $CD4^+$, $CD8\alpha^+$, and $CD8\beta^+$ lymphocytes, which probably reflects the increased percentages of $Gr\text{-}1^+$ $CD11b^+$ granulocytes in the peripheral blood samples of uninfected mice compared to those for the PBS-treated controls (Fig. 5).

PBS-injected mice with microfilariae showed significantly

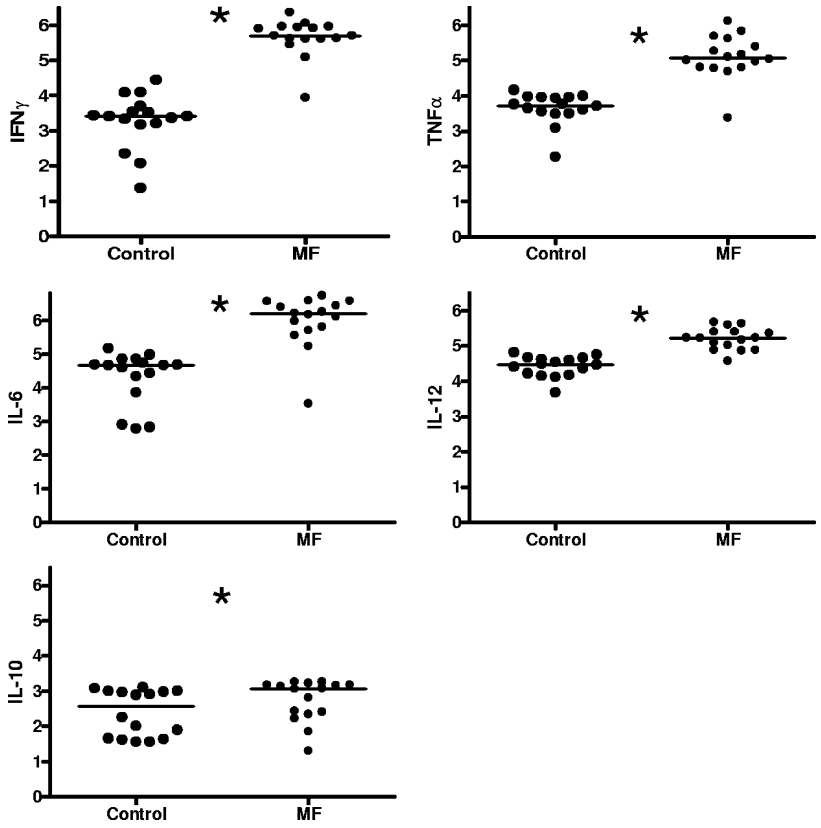


FIG. 3. Cytokine production [log (pg/ml + 0.5)] of microfilaria-infected C57BL/6 mice ($n = 16$) and noninfected controls ($n = 16$) 6 hours (IFN-γ, IL-6, IL-12, and IL-10) and 1 hour (TNF-α) after LPS challenge. Shown are the results from three independent experiments. Significant differences were analyzed by the Mann-Whitney U test (*, $P < 0.05$).

increased numbers of Gr-1$^+$ CD11b$^+$ granulocytes, CD49b$^+$ NK cells, and CD11b$^+$ Gr-1$^-$ monocytes ($P = 0.06$) and decreased numbers of CD8α$^+$ and CD8β$^+$ T cells compared to the uninfected PBS controls (Fig. 5).

**Decreased body temperature in septic mice with microfilaremia.** Six hours after LPS challenge, mice infected with microfilariae developed lower body temperatures (median 28°C) than uninfected mice challenged with LPS (median, 32°C) (Fig. 6 A). Similarly, mice that had been implanted with adult female worms from patent infections that were actively releasing microfilariae into the circulation developed greater drops in temperature after LPS challenge than wild-type mice and tended to have lower temperatures than mice implanted with either adult male worms or adult female worms from postpatent infections (Fig. 6B). Microfilaria-infected mice deficient in IFN-γR and TNFR1 developed less-severe hypothermia after LPS challenge than microfilaria-infected immunocompetent mice, with median core body temperatures of 30°C to 33°C 6 hours after LPS challenge (Fig. 6A). There were no statistically significant differences in body temperature between microfilaria-infected MARCO knockout mice and wild-type mice after LPS injection (Fig. 6A).

TABLE 1. Cytokine and chemokine concentrations 6 hours after LPS or PBS injection of C57BL/6 mice[a]

| Chemokine or cytokine | Median concn (10th percentile/90th percentile) (pg/ml) for indicated treatment | | | |
|---|---|---|---|---|
| | Mf + LPS | Control + LPS | Mf + PBS | Control + PBS |
| MIP-1α | **2,060** (682/4,362) | **171** (104/239) | 28 (24/33) | 28 (14/43) |
| MIG | **11,800** (10,020/14,110) | **1,910** (1,474/3,607) | **878** (337/1,730) | **0** (0/11) |
| MCP-1 | **25,750** (19,160/28,000) | **5,840** (4,232/6,772) | 11 (11/92) | 16 (11/23) |
| GMCSF | **249** (184/489) | **50** (25/83) | 0 (0/3) | 0 (0/18) |
| IL-1α | **1,225** (394/1,756) | **26** (0/57) | 0 (0/0) | 0 (0/0) |
| IL-1β | **37** (13/105) | **0** (0/0) | 0 (0/0) | 0 (0/0) |
| IFN-γ | **499,053** (201,819/1,028,681) | **2,563** (170/12,100) | **607** (361/831) | **0** (0/6) |
| IL-12p40 | **166,442** (73,986/408,245) | **29,798** (13,339/51,009) | **3,579** (2,009/8,522) | **945** (0/2,761) |

[a] There were 8 mice per group, except for IL-12p40 and IFN-γ testing, in which there were 15 mice per group. Mice were infected with microfilariae (Mf) or sham treated (control). Significant differences ($P < 0.05$) between uninfected and infected animals injected with either LPS or PBS were analyzed by the Wilcoxon/Kruskal-Wallis test and are presented as bold numbers.

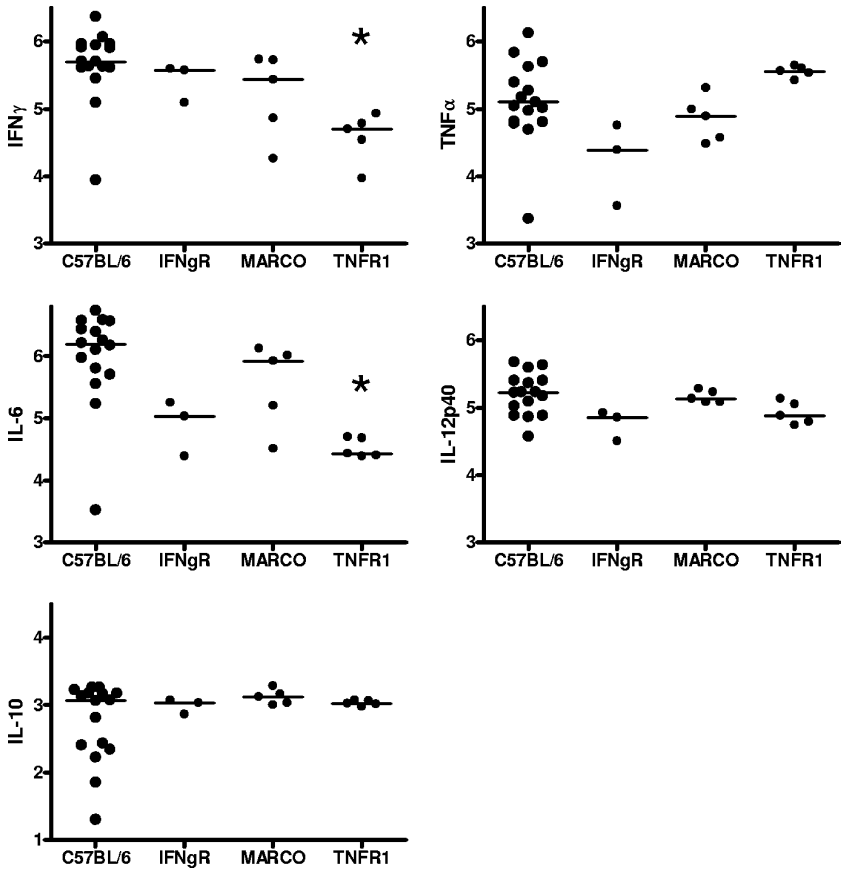


FIG. 4. Cytokine production [log (pg/ml + 0.5)] of microfilaria-infected C57BL/6 ($n = 16$), IFN-gR ($n = 3$), MARCO ($n = 5$), and TNFR1 ($n = 5$) knockout mice 6 hours (IFN-γ, IL-6, IL-12, and IL-10) and 1 hour (TNF-α) after LPS challenge. Shown are the results obtained from two independent experiments. Significant differences between knockout mice and the C57BL/6 control group were analyzed by the Kruskal-Wallis test, followed by Dunn's post hoc multiple comparisons (*, $P < 0.05$).

## DISCUSSION

Pathogenic helminths have the ability to downregulate the immune responses of their hosts (14, 26). Indeed, helminths have been shown to ameliorate autoimmune diseases in several animal studies (24, 27, 34, 41), and in humans, egg preparations of the pig whipworm (*Trichuris suis*) have been used successfully to treat severe inflammatory bowel disease (35).

Whereas most of these studies have focused on the effects helminths have on the adaptive immune response, in this study we evaluated the effects helminths have on the innate immune system. Through the use of an LPS sepsis model, we have demonstrated that helminths substantially alter innate immune responses and that the type of immune modulation observed is specific to the stage of the parasite.

One of the most striking findings in our study is that female adult worms from prepatent infections (microfilaria negative) seemed to diminish the inflammatory response induced by LPS challenge. Implantation of female adult worms from prepatent infections resulted in decreased concentrations of the proinflammatory cytokines IFN-γ, TNF-α, and IL-12p40 in the peripheral blood samples of mice compared to the levels for sham-treated controls after LPS challenge. Therefore, we suggest that the implanted worms initiated an immunosuppressive milieu in mice which dampened the immune response to LPS. Possible mechanisms for this phenomenon include induction of higher frequencies of regulatory T cells, development of alternatively activated macrophages, diminished antigen presentation, and a more pronounced Th2 immune response. All of these mechanisms are known to be provoked by helminth infections, and all have the potential to reduce proinflammatory immune responses to LPS. Another possible explanation for the protective effects that prepatent female adult worms had on LPS challenge is the release of helminth products that directly impede the immune response triggered by LPS, as earlier studies have demonstrated that helminths can secrete products that reduce the responses of Toll-like receptors (TLRs) to LPS. Soluble extracts from *Brugia malayi* and *Schistosoma mansoni* reduced the TLR4 expression on macrophages and the ability of TLRs from dendritic cells to respond to LPS (21, 31, 38). Further studies with female adult *L. sigmodontis* worms will investigate these possible protective mechanisms with respect to LPS-induced sepsis.

Interestingly, the implantation of female adult worms from postpatent infections (adult female worms which no longer release microfilariae into the peripheral blood samples of their hosts) or of male adult worms seemed not to decrease the proinflammatory response induced by LPS challenge. The loss of the beneficial effect seen in mice infected with female adult

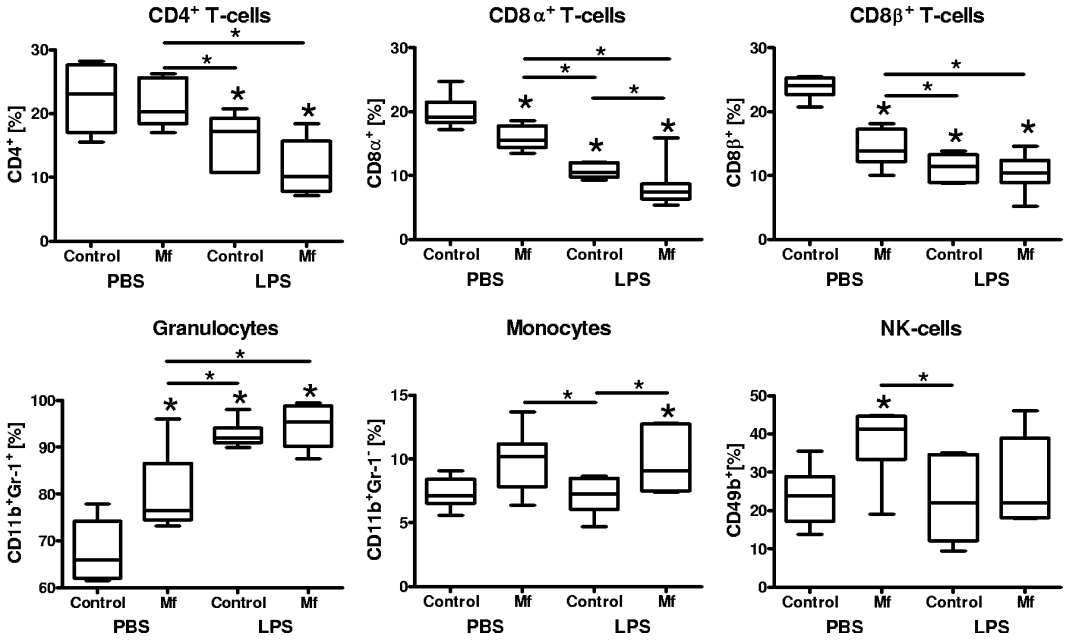


FIG. 5. Cell populations in the peripheral blood samples of C57BL/6 mice ($n = 7$ per group) treated with microfilariae (Mf) and LPS or microfilariae and PBS as well as the corresponding sham-treated controls ("Control" in LPS and PBS groups). Significant differences were determined by the Wilcoxon/Kruskal-Wallis test after stabilization of data by arcsinus transformation [arcsin (square root of values in percentage/100)]. Significant differences between the PBS-injected control group and the other groups are displayed with a single star, and horizontal bars with stars indicate any additional significant differences (*, $P < 0.05$).

worms from prepatent infections after LPS challenge was probably due to the fact that the female adult worms from postpatent infections still released a few microfilariae, which were detected in peritoneal smears but not in the peripheral blood. Alternatively, this finding could be due to the higher ages of the worms and decreasing abilities of immunomodulation. While we can only speculate, the lack of a protective response from adult male worms suggests that perhaps adult female worms release immunomodulatory substances to protect microfilariae from immune destruction.

In contrast to the observed protective effects associated with implantation of adult female worms from prepatent infections, the presence of microfilariae in the peripheral blood, following either direct injection or implantation of microfilaria-releasing female adult worms, resulted in a marked reduction of survival after LPS challenge. This increased mortality after LPS challenge appears to be due to an augmentation in the inflammatory response toward LPS in the setting of microfilariae. Specifically, the presence of microfilaremia was associated with increased levels of proinflammatory cytokines and chemokines and with a greater reduction in body temperature in response to LPS challenge than for uninfected mice.

After LPS challenge, concentrations of TNF-α and IL-1, which play central roles in the innate immune response to LPS (2, 10), were significantly greater in microfilaria-infected mice than in control mice. In addition, the most abundant cytokine

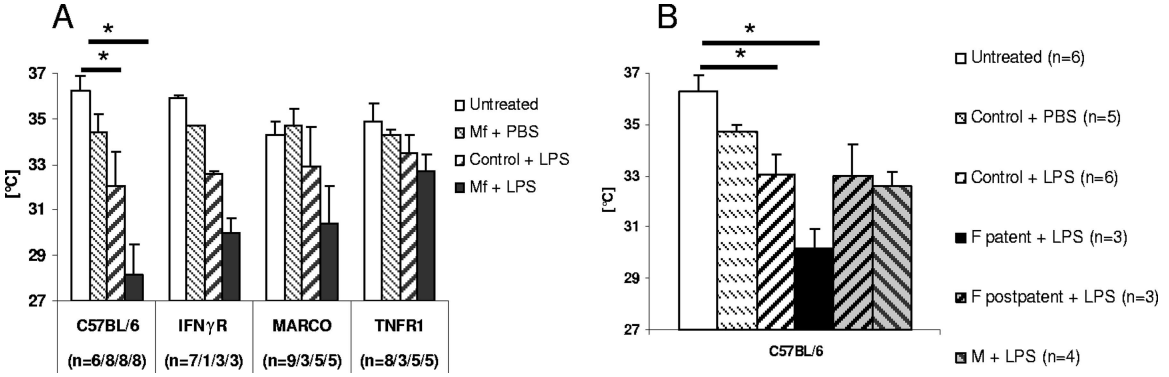


FIG. 6. Body temperature 6 hours after injection of LPS or PBS in C57BL/6, IFN-γR, MARCO, and TNFR1 knockout mice which were infected with microfilariae (Mf) or were sham treated (Control) (A) and C57BL/6 mice implanted with male (M) or female (F) adult worms from patent or postpatent infections and sham-treated controls (B). Shown are medians plus 90th percentiles. Significant differences were analyzed by the Kruskal-Wallis test, followed by Dunn's post hoc multiple comparisons (*, $P < 0.05$).

in the peripheral blood samples of microfilaremic mice after LPS injection was IL-6, which enhances the acute-phase response of the liver and is a marker for sepsis that correlates with severity of inflammation (28).

The fatal course of disease observed when microfilaremic mice were given a sublethal dose of LPS may be due to IFN-γ and IL-12 induced by microfilariae. A single injection of microfilariae resulted in a Th1-type immune response characterized by elevated numbers of monocytes, granulocytes, and NK cells in the peripheral blood and higher plasma concentrations of IFN-γ, IL-12, and MIG. These results are similar to those reported earlier by Lawrence et al. for *B. malayi* (25), a related filarial nematode. In *B. malayi* infections, a serine protease inhibitor (serpin, BM-SPN-2) was shown to be partly responsible for IFN-γ production by host cells (42), suggesting that in our model specific microfilarial antigens may have been responsible for increases in IFN-γ.

The elevated levels of IFN-γ could also have been driven by *Wolbachia* organisms, intrahelminthic bacteria present in *L. sigmodontis*. These endosymbiotic organisms, which have been shown to be released along with microfilariae by mature worms or by dying microfilariae, induce proinflammatory immune responses (11, 17). Further, the *Wolbachia* surface protein WSP has been shown to induce IL-12 production by acting through TLR2 and -4 (4, 5). However, fatal effects mediated by microfilaria-derived components other than *Wolbachia* products have been demonstrated using filarial species that do not contain *Wolbachia* endosymbionts (29). In addition to direct stimulation of cytokine production by microfilariae or *Wolbachia*, it is also possible that the higher concentrations of IL-6, IL-12, TNF-α, MCP-1, and MIP-1α observed in LPS-challenged mice infected with microfilariae were due to the elevated numbers of monocytes induced by the microfilaremia.

In summary, we think that the lethal outcome of LPS-induced sepsis in microfilaria-positive mice was due to increased numbers of monocytes, granulocytes, and NK cells in the peripheral blood induced in response to the microfilariae. In addition, IFN-γ, which was elevated either due to *Wolbachia* or due to the microfilaria itself, is able to upregulate the expression of TLR4 on monocytes, increase phosphorylation of IL-1 receptor-associated kinase, and enhance the DNA-binding capacity of NFκB, all of which results in increased IL-12 and TNF-α secretion by monocytes (3). These actions further activate monocytes, natural killer cells, and neutrophils (2) and can also be contributing to the strong LPS-induced inflammatory reaction observed in microfilaria-infected mice.

The usage of IFN-γR and TNFR1 knockout mice showed that the lethal course caused by LPS injection in microfilaria-infected mice was mediated by IFN-γ and TNF-α. Although we performed only a single experiment, with just three microfilaria-infected IFN-γR knockout mice, they showed the lowest measured cytokine levels after LPS challenge and relatively little decrease in body temperature, which likely enabled survival. Similarly, the survival rate of TNFR1 knockout mice infected with microfilariae was significantly greater than that of control microfilaria-infected mice.

The defect of MARCO, an LPS binding type A scavenger receptor expressed on macrophages, dendritic cells, and microglia cells (15), seemed not to ameliorate the course of disease after LPS challenge of microfilaria-infected mice. Microfilaria-infected MARCO knockout mice had neither significantly increased body temperatures nor significantly decreased concentrations of IFN-γ, TNF-α, IL-12p40, and IL-6 compared to the immune-competent mice after LPS challenge, resulting in the deaths of three out of five infected MARCO knockout mice after LPS challenge. These findings suggest that MARCO may have a slight influence on LPS-induced sepsis but that other surface molecules that sense LPS, like TLR4, CD11b/CD18, and ion channels (10), may be responsible for the observed lethal outcome after LPS injection.

The fatal outcome observed in our microfilaria-infected mice and in our animals implanted with female worms from patent infections does not mirror the expected outcome of sepsis under the conditions of a natural filarial infection. In contrast to our implantation experiments where microfilariae are released immediately in large numbers, the time course following natural infection allows the developing worms to down-modulate the immune response before they release microfilariae, thereby preventing an overwhelming immune response at this life cycle stage. When this regulated condition cannot be achieved, severe pathology, such as sowda in onchocerciasis or tropical pulmonary eosinophilia in lymphatic filariasis, may occur. While *L. sigmodontis* infection in mice does not model the lymphatic damage observed in human filariasis, as the *L. sigmodontis* adults reside in the pleural space and not in the lymphatics, it can be speculated that the presence of worms and their immunomodulatory effects may affect the outcome of disease in lymphatic filariasis as the modulation of the immune response by adult female worms from prepatent infections may reduce inflammatory responses in the host. The observation of two polar groups in lymphatic filariasis (30), with one group having abundant microfilariae in circulation, but no symptoms or chronic pathology, and another group having elephantiasis and an absence of microfilariae, may therefore be possibly explained by successful immunosuppression by the helminths in the first group and a possible lack of immune modulation in the second.

Pathological effects due to a failure of immune down-regulation have also been observed in coinfections of BALB/c mice with *L. sigmodontis* and *Plasmodium chabaudi* where severe malaria occurred in animals that did not develop a patent filarial infection (13). Moreover, these animals showed high IFN-γ levels, indicating a strong Th1-type response. Although the authors did not state whether the strong immune response observed in some of the coinfected animals was the cause or the consequence of the absence of circulating microfilariae, one can speculate that in mice with patent filariasis down-modulation of the immune response by female filariae may facilitate establishment of microfilaremia as well as prevent severe clinical manifestations of malaria.

Because our study utilized a model of LPS injection to study sepsis, we cannot conclude that infections with female adult worms from prepatent infections would protect against bacterium-induced sepsis. Theoretically, a downregulation of inflammatory responses by helminth parasites could be associated with an increased susceptibility to proliferating bacteria. Such a phenomenon was observed in coinfection studies with the Th2-inducing rodent helminth *Heligmosomoides polygyrus* and the bacterium *Citrobacter rodentium* (7, 8).

In summary, we have demonstrated that different filarial life

cycle stages of the filaria *L. sigmodontis* have differential impacts on LPS-induced inflammation in a murine sepsis model. While premature adult worms diminished the inflammatory response induced by LPS challenge in mice, microfilariae on the other hand worsened the course of LPS challenge. The field of immune modulation by helminths is an active one, with several research groups seeking to find which helminth antigens are responsible for immune modulation and some current studies even demonstrating success in treating autoimmune diseases with helminths. On the basis of our findings in this study, we suggest that future work for trying to determine the antigens responsible for helminth-induced downregulation should focus on molecules expressed in adult female worms and not in microfilariae.

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